

## **Wild Rice Juvenile Seedling Growth Test: Anoxic Conditions**

Experimental Method for Hydroponic Sulfide Toxicity Testing in Anoxic Conditions Using  
*Zizania palustris* (Wild Rice) as Test Organism

### **1.1 Scope and Application**

- 1.1.1 This method describes procedures developed by Dr. John Pastor and Brad Dewey of the University of Minnesota – Duluth Biology Department, in coordination with MPCA research scientists, for the purposes of conducting an aquatic toxicity test to determine the response of *Zizania palustris* (Wild Rice) juvenile seedlings exposed to various concentrations of dissolved sulfide in an anoxic (oxygen free) hydroponic growth medium.
- 1.1.2 This method consists of exposing *Z. palustris* juvenile seedlings in a hydroponic growth medium to different dissolved sulfide concentrations via a four-step dilution series with replication. Juvenile seedlings that are exposed to growth medium without the addition of sulfide are used as a negative control and a baseline of comparison.

### **1.2 Summary of Method**

- 1.2.1 Juvenile seedlings of the aquatic macrophyte, *Z. palustris*, are exposed to a dilution series of dissolved sulfide concentrations within a statically-renewed anoxic hydroponic system. A complete toxicity test consists of 10 days of juvenile seedling exposure, after which the juvenile seedling biomass and vegetative growth are measured and recorded as parameters of response and comparison (see Section 1.10: *Phytotoxic Effects*). Measuring the response of the juvenile seedlings after this 10 day exposure to a particular dissolved sulfide concentration is conducted as statistical comparison of differences in the growth parameters between a treatment level and control and between different treatment levels.

### **1.3 Quality Control Considerations**

- 1.3.1 Toxic substances may be introduced by contaminants in dilution water, sampling hardware, or testing equipment.
- 1.3.2 Adverse effects of pH changes and cationic constituents in test media may augment or mask adverse effects of toxic substances.
- 1.3.3 Improper sampling of test media may adversely affect test results (see section 1.5, Standards and Reagents, and section 1.6, Toxicity Test Procedures)
- 1.3.4 Additional details are found in the document titled: “*Wild Rice Sulfate Standard Hydroponic Experiment on Response of Wild Rice to Sulfide - Quality Assurance Project Plan*”

- 1.3.5 Maintaining an anoxic environment or preventing the introduction of compounds that may react with the dissolved sulfide in the test media is necessary for maintaining consistent sulfide concentrations over a test period. All aspects of this method were designed with this consideration in mind.

#### **1.4 Necessary Apparatus and Materials**

- 1.4.1 Seeds of *Z. palustris* are prepared in the laboratory for germination (see section 1.7: *Wild Rice Seed Preparation*). To initiate exposures, sufficient numbers of germinated seeds (sprouts) must be available. Each test container (jar) contains 7 sprouts.
- 1.4.2 Environmental Growth Chamber: Temperature control range of 15° C to 30° C  $\pm$  1°C). Juvenile growth tests are performed in light/dark intervals of 16 hours of light and 8 hours of dark.
- 1.4.3 Test chambers: 700 mL borosilicate glass jars capped using phenolic screw caps with chlorobutyl septa (Wheaton).
- 1.4.4 Meter: pH for routine physical measurements.
- 1.4.5 Volumetric flasks and graduated cylinders: class A, 10 – 2000 mL borosilicate glass for preparation of test media.
- 1.4.6 Volumetric pipets
- 1.4.7 Glass pipets
- 1.4.8 Pipet bulbs and fillers
- 1.4.9 Balance: analytical, capable of accurately weighing 0.1 mg.
- 1.4.10 Magnetic stirrer and stir bars: for mixing test and growth media.
- 1.4.11 Filtering apparatus: for membrane and /or glass fiber filters.
- 1.4.12 Tape: for labeling test chambers and jars for treatment and replicate identification.
- 1.4.13 Water purification system: deionized water or equivalent.
- 1.4.14 20 L glass carboy for preparing Hoagland's solution.
- 1.4.15 "High Purity" grade compressed nitrogen gas cylinder with attached oxygen scrubber and hose for degassing test media.

#### **1.5 Standards and Reagents**

- 1.5.1 Reagent-grade chemicals are used to prepare hydroponic growth media, which consists of a modified Hoagland's nutrient solution.

- 1.5.2 A modified 1/5 concentration Hoagland's stock nutrient solution, as determined beneficial for wild rice growth, is prepared as part of the test media from a 1/2 concentration stock Hoagland's solution, or more often as needed from a 1.0 M stock solution. (see Table 1. for 1/5 Hoagland's stock solution nutrient composition and concentrations)
- 1.5.3 PIPES buffer is added to the test media to maintain consistent pH levels throughout an experiment.
- 1.5.4 Stock sulfide solutions (20 – 30 mM) are prepared as needed by adding  $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$  (Sodium Sulfide Hydrate) to deionized and degassed water.
- 1.5.5 The hydroponic media is degassed and made anoxic in preparation for use in test media by bubbling "High Purity" grade nitrogen gas with an attached oxygen scrubber through the solution for at least 24 hours.
- 1.5.6 Reagent water: defined as deionized water that does not contain substances that are toxic to the test organisms.
- 1.5.7 Appropriate amounts of each test media (700 mL/treatment replicate jar) multiplied by the number of replicates, plus an additional amount required for a sample to be used to characterize the chemistry of the stock test media (approx. 2100 mL per treatment for 3 replicates and ~200 mL sample for chemistry characterization) are made up immediately before starting a test and every day of test media renewal using predetermined amounts of 1/5 strength Hoagland's, PIPES buffer (Piperazine-N,N'-bis(2-ethanesulfonic acid) sesquisodium salt, Fisher Scientific/Acros Organics # AC32778-5000) and calcium carbonate. The pH is adjusted to 6.8 +/- 0.2 with 1 M HCl.

Table 1: Composition of Hydroponic Growth Media

Compound	Molar concentration in 1/5 <sup>th</sup> strength growth solution
MgCl	0.4 mM
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	2.0 mM
KCl	1.0 mM
NH <sub>4</sub> Cl	0.16 mM
KH <sub>2</sub> PO <sub>4</sub>	0.026 mM
H <sub>3</sub> BO <sub>3</sub>	22.5 µM
MnCl · 4 H <sub>2</sub> O	4.5 µM
ZnSO <sub>4</sub> · 7 H <sub>2</sub> O	0.5 µM
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	0.15 µM
MoO <sub>3</sub>	0.07µM
Fe-EDTA	45.0 µM
Na <sub>2</sub> SiO <sub>3</sub> · 9H <sub>2</sub> O	1.5 mM
PIPES buffer	5.0 mM
CaCO <sub>3</sub>	

## 1.6 Toxicity Test Procedures: Toxicant Exposures

- 1.6.1 Each toxicity test will consist of at least four toxicologically relevant test concentrations of sulfide in the hydroponics test media (hydroponics growth medium + sulfide) and a negative control which consists of the hydroponics growth medium without any sulfide added.
- 1.6.2 Each of the four exposure concentration levels and control are replicated three times, using the same size 700 ml glass jars for each set of replicates.
- 1.6.3 Germinated wild rice seed (sprouts) as described in section *1.7 Wild Rice Seed Preparation*, are used to initiate the toxicity test.
- 1.6.4 A total of 7 sprouts are selected and removed individually from a pool of conditioned seeds (section *1.8 Test Organisms*) using a light forceps and are placed into a uniquely numbered 700 ml glass jar not yet associated with a treatment level or replicate. This sprout placement procedure is conducted using a randomization scheme, where a seed is placed into a jar corresponding to the first integer read from a random integer table. This randomization is done for all jars prepared for testing until 7 sprouts occupy each test jar.
- 1.6.4 Each jar is labeled with tape using a unique descriptor for describing the particular concentration level of sulfide in the test media and replicate for that jar.
- 1.6.6 The sprouts and jars are place in a nitrogen tent for about two hours to assure anoxic conditions. Each labeled jar is filled to the top with the prepared (anoxic) hydroponic growth media and capped. The jars are then securely recapped.

- 1.6.7 Exposure jars are spiked with a volume of concentrated sulfide media appropriate for the corresponding nominal sulfide concentration of that jar. Control exposure jars are spiked with degassed distilled water to simulate treatment spiking. Sulfide solutions are spiked into the jars through the chlorobutyl septa in the cap using Hamilton volumetric glass syringes. Spike volumes range between 0.2 – 3 mL of spike solution depending on target exposure concentrations and nominal concentration of stock sulfide solution concentration.
- 1.6.10 The jars are inverted twice to mix the test media and are placed onto a tray.
- 1.6.11 The experimental jars, now each containing the sprouts, hydroponic test media, and sulfide exposure, are placed onto a tray and stored in a growth chamber with a light/dark schedule (see section 1.9) for the duration of the 10 day experiment, with the exception of being taken out of the growth chamber for the renewal of test media.
- 1.6.12 Test media in the jars are renewed every two (2) days.
- 1.6.13 During a test media renewal day, the caps of the jars are first removed to allow collection of a sample for the immediate measurement of pH in the old test media. Additional samples are then taken of the old test media and preserved for analytical chemistry analysis of sulfide concentration. Proper sampling techniques, preservation methods, and sample labeling procedures are followed as required by the laboratory contracted for analysis. Any remaining old test media not used for pH or analytical chemistry is gently poured off, leaving approximately one vertical cm of old media in the bottom of the jar.
- 1.6.14 The addition of new hydroponic test media to jars is completed by first degassing the empty jar using a flow of nitrogen sufficient to replace three jar volumes (about 2100 mL). This is completed for each jar as is it emptied. Newly prepared test media is added by siphoning from the container of stock solution until it reaches the top of the jar. This occurs as the jar is continuing to be degassed. The screw cap is then replaced on the jar.
- 1.6.15 Chemistry as described in the section *1.12: Analytical Chemistry*.
- 1.6.16 Duration of the experiment is 10 days with test media renewals occurring on 4 of the 10 days.

## 1.7 Wild Rice Seed Preparation

- 1.7.1 Wild rice seed must undergo a conditioning phase following its harvest from the field. In the wild, wild rice drops into the water after the seed has ripened, and sinks to the sediment. This seed, if left undisturbed, stays on or just below the surface of the sediment over the winter. This cold phase serves to condition the seed to enable it to germinate once water temperatures increase in the spring.

- 1.7.2 The following is a procedure that describes the method and handling of wild rice seed from initial harvest to its use in juvenile growth toxicity tests.
- 1.7.3 Freshly harvested seed should be kept cool and moist and be placed into storage as soon as possible after field collection.
- 1.7.4 Harvested seed prepared for storage can be kept a) in plastic zip lock bags in a cooler set at just above freezing (4° C), or b) submerged in water just above freezing in the dark. Seed stored in either manner can have satisfactory germination rates for one to two years.
- 1.7.5 To begin the seed conditioning for germination, an aliquot of seed (approximately 2000 seeds) is removed from this 'dry' cold storage (as described in option (a) in 1.7.3) and placed into a container with water kept submerged at near freezing temperatures for at least one month. Following this time period, seed is ready (or conditioned) for germination for at least several months. For purposes of use in laboratory testing, seed set in this conditioning phase is kept for up to two months before a fresh aliquot of seed is brought into the conditioning phase. Use of storage option (b) keeps the seed in this wet, cold conditioned phase until needed for testing. See Image 3.

## **1.8 Test Organisms, Germinated Wild Rice Seed**

- 1.8.1 As seed is needed for testing, a smaller aliquot from the wet-stored (conditioned seed) is removed and placed into a container with water and placed in the dark incubator at 20+/- 1C. Seed used for initiating a toxicity test are visually screened for viability based on the color of the seed coat and fullness of the seed body. Seeds that float, are misshaped, or are otherwise malformed are not used for testing.
- 1.8.2 For the juvenile growth test method, the seed will begin to sprout in about 2 to 3 days. Germinated seeds (referred to as a sprout) are selected with at least 1-2 cm of mesocotyl growth. A germinated seed (sprout) is described as growth of the mesocotyl that is longer than the seed coat. See Image 4.
- 1.8.3 Sprouts are selected from the pool of available seeds and placed into a separate container with water. A total of 126 sprouts are selected with 105 sprouts used for initiating the toxicity tests, and 21 sprouts put aside to be dried and weighed to measure initial weight.

## **1.9 Light, Photoperiod, Temperature and Humidity Test Conditions**

- 1.9.1 Tests are performed under a 16h:8h light/dark schedule.
- 1.9.2 Light intensity is 350  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  as measured at the mid-point of the exposure jar.
- 1.9.3 Temperature is maintained at 21° C during lighted periods and 19° C during dark period.

- 1.9.4 The Test growth chamber is maintained at 85% humidity.

## **1.10 Phytotoxic Effects**

- 1.10.1 Observations of sprouts should be made during test media renewal every 2 days. All abnormalities should be recorded.
- 1.10.2 Observations should include the date, time, treatment level, and replicate number.
- 1.10.3 After the duration of the test, exposure jars are kept in the random order as placed initially.
- 1.1.4 Old test media are decanted into labeled sample bottles in the same manner as described for test media renewal.
- 1.10.5 Once the test media is removed, the sprout is removed from the jar for subsequent measurements.
- 1.10.6 Measures of stem/leaf length (mesocotyl growth) are performed by placing the stem/leaf stretched out on a flat surface next to ruler with the zero mark aligned with the point of stem-root transition. The length from the stem-root transition to the tip of the stem/leaf is measured and recorded to the nearest millimeter. In Addition, a measurement is recorded of the longest root developed on the seedling. See Image 6.
- 1.10.7 Plant biomass is measured as follows: Each seedling is placed into a numbered, weighed aluminum weighing dish (Fisher 08-732-101). The dishes are placed on trays, and the trays are put into a drying oven at 100° C for at least 48 hours. Each dry sample and dish is placed into a desiccator until in equilibrium with the ambient room temperature. Dry samples + pre-weighted dishes are weighed to the nearest 0.1 mg.
- 1.10.8 All dried plant materials are weighed on a Sartorius 2700 balance to 0.1 mg following the method titled “Total Plant Biomass (Dry Weight) Methods” found in Appendix C of the document titled: “Hydroponic Experiment on Response of Wild Rice to Sulfide- Quality Assurance Project Plan”. See Image 9.

## **1.11 Acceptability of Test Results**

- 1.11.1 At least 90% of negative control juvenile seedlings are living at test termination.
- 1.11.2 Mean mesocotyl length of juvenile seedlings from control exposures must be at least 5.0 cm at the end of the 10 day duration of growth.
- 1.1.3 Control juvenile seedlings should not indicate any visible phytotoxic or developmental symptoms at any time during the test.

## 1.12 Analytical Chemistry

- 1.12.1 The sampling collection and handling activities described below are consistent with the methods and procedures of the Minnesota Department of Health's Environmental Laboratory. These are subject to change as different laboratories may require different sampling collection and handling methods.
- 1.12.2 Sampling and analysis of prepared test media used for initiating and renewing test exposures will use the following procedures.
- 1.12.3 Initial and renewal test media –Immediately after adding the test media into the jars an aliquot (approximately 250 ml) of the remaining unused portion is poured directly into a pre-labeled sample bottle and preserved as appropriate for the type of analysis requested.
- 1.12.4 Old test media – Before exchanging renewal test media or before decanting the final media, the jar is inverted to adequately mix the test solution. A syringe is injected into the jar cap. A slight positive pressure is added into the jar using “high purity” nitrogen gas pushed through a separate syringe line to allow for approximately 30 mL of test media to flow out. The line is then attached to a 125 mL evacuated serum jar containing zinc acetate for the collection and preservation of the sample for sulfide analysis, as instructed by the Minnesota Department of Health - Environmental Laboratory's method.
- 1.12.5 Samples of the initial, old and renewal test media are collected, labeled, preserved, stored and transported to the Minnesota Department of Health Environmental Laboratory for sulfide quantification per instructions supplied by the laboratory.

## References

- D. R. Hoagland and D.I. Arnon. 1950. The water-culture method for growing plants without soil. Circ. 347. Univ. of Calif. Agric. Exp. Station, Berkley
- U.S. EPA. 2012. Ecological Effects Test Guidelines. OCSPP 850.4230: Early Seedling Growth Toxicity Test. EPA 712-C-010.
- U.S. EPA. 2012. Ecological Effects Test Guidelines. OCSPP 850.4100: Seedling Emergence and Seedling Growth. EPA 712-C-012.
- U.S. EPA 2012. Ecological Effects Test Guidelines. OCSPP 850.4400: Aquatic Plant Toxicity Test Using *Lemna* spp. EPA 712-C-008.
- U.S. EPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4<sup>th</sup> ed. EPA-821-R-02-013.





Image 1. Random placement of exposure tubes.



Image2. Exposure solution renewal



Image3. Wild rice seed conditioned for germination.

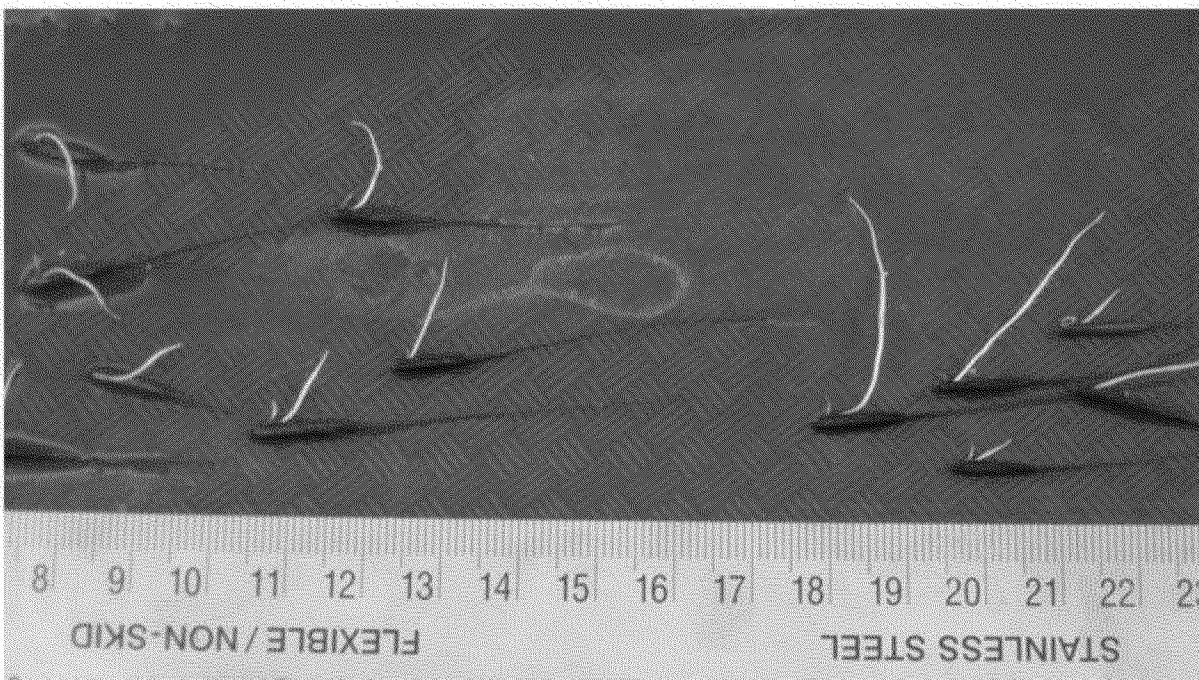


Image 4. Wild rice sprout selection; sprouts on the left have mesocotyl growth of 1-2 mm long, sprouts on the right have mesocotyl growth either too long or too short to be acceptable for test initiation.

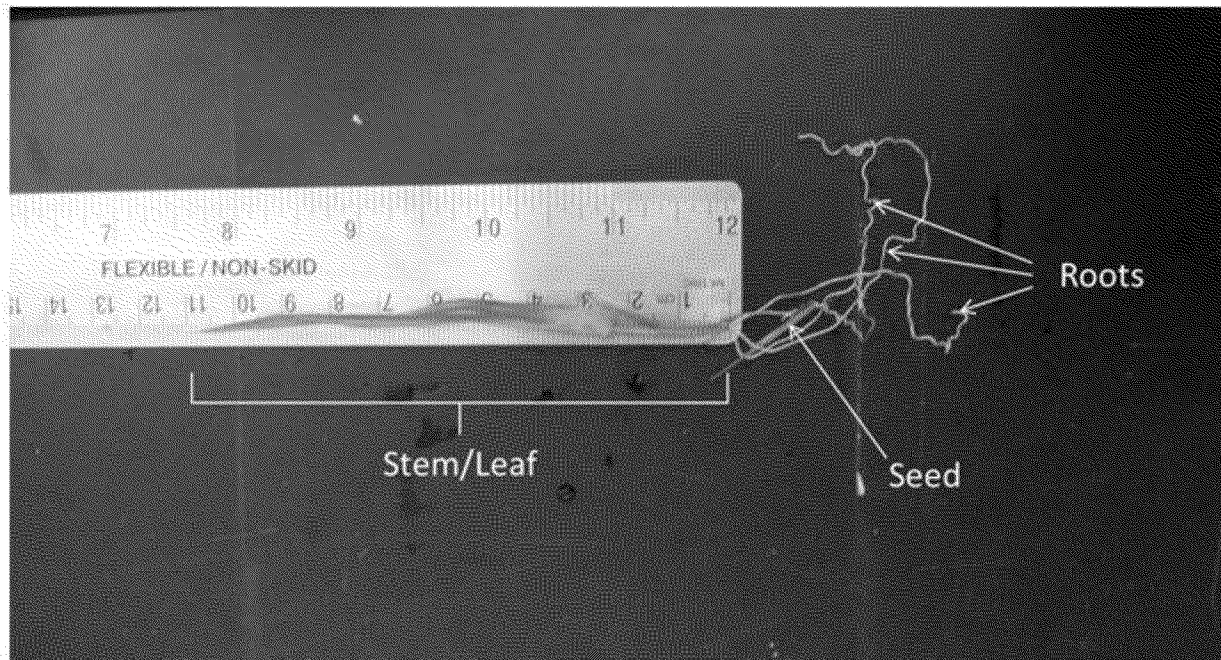


Image 5. Identification of sections of wild rice sprout.

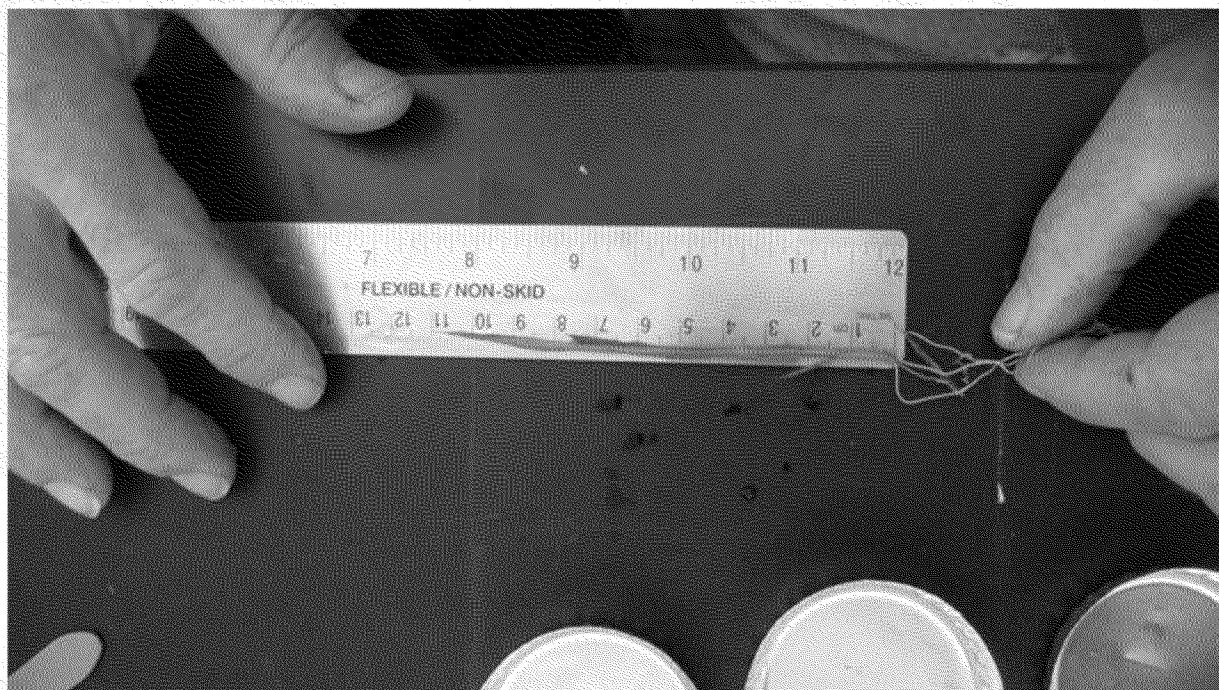


Image 6. Wild rice seedling length measurement following 10 d exposure period.



Image 7. Wild rice seedling showing removal of stem portion in preparation for drying.

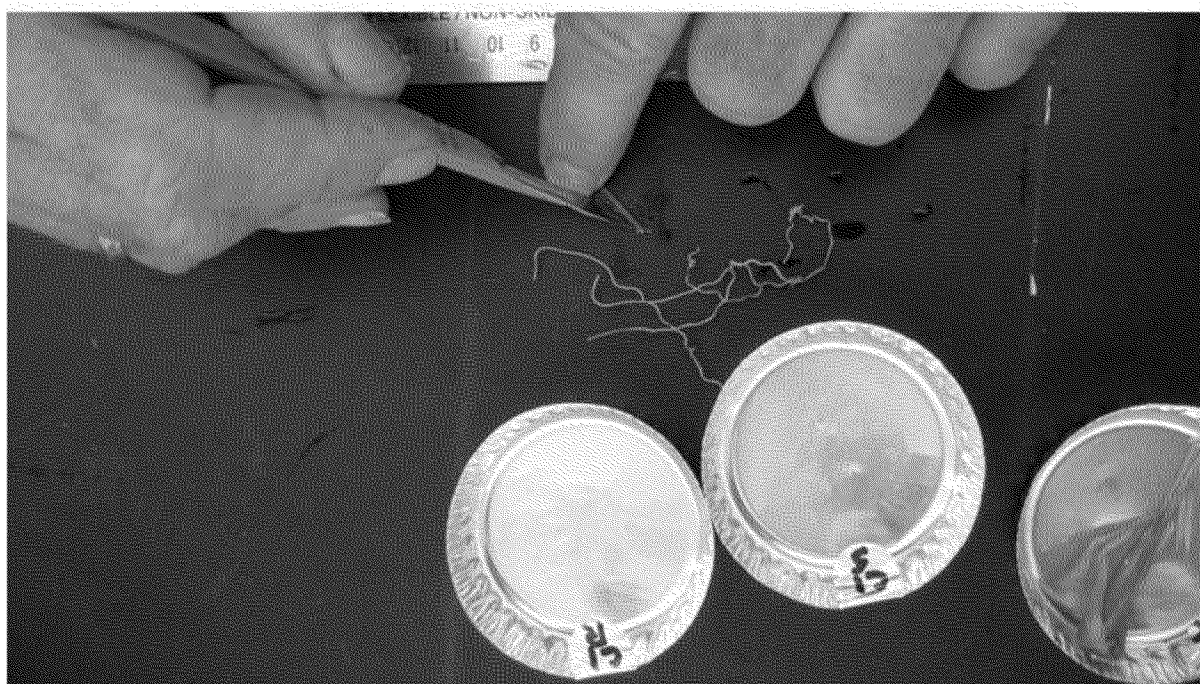


Image 8. Wild rice seedling root removal showing roots being cut from remaining seed material in preparation for measuring root length and area; stem portion of seedling is shown in a drying pan.



Image 9. Wild rice seedling showing stem, root and remaining seed portions in preparation for drying.